Immunity
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Suppression of Phosphatidylinositol 3,4,5-Trisphosphate Production Is a Key Determinant of B Cell Anergy

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DOI 10.1016/j.immuni.2009.08.026

SUMMARY

Anergy is a critical physiologic mechanism to censor self-reactive B cells. However, a biochemical understanding of how anergy is achieved and maintained is lacking. Herein, we investigated the role of the phosphoinositide 3-kinase (PI3K) lipid product PI(3,4,5)P_3 in B cell anergy. We found reduced generation of PI(3,4,5)P_3 in anergic B cells, which was attributable to reduced phosphorylation of the PI3K membrane adaptor CD19, as well as increased expression of the inositol phosphatase PTEN. Sustained production of PI(3,4,5)P_3 in B cells, achieved through conditional deletion of *Pten*, resulted in failed tolerance induction and abundant autoantibody production. In contrast to wild-type immature B cells, B cell receptor engagement of PTEN-deficient immature B cells resulted in activation and proliferation, indicating a central defect in early B cell responsiveness. These findings establish repression of the PI3K signaling pathway as a necessary condition to avert the generation, activation, and persistence of self-reactive B cells.

INTRODUCTION

A large proportion of newly formed B cells express antigen receptors possessing some degree of self-specificity. Hence, the body is challenged with the necessity of providing an ample pool of naive B cells capable of responding to diverse antigenic challenges while preventing the release and/or activation of self-reactive B cells. The consequences of self-antigen encounter is governed by B cell receptor (BCR) signaling and can be influenced by additional microenvironmental factors such as T cell-derived factors, B cell-activating factor (BAFF) and Toll-like receptor (TLR) ligands. The nature of the BCR signal has been well described in terms of "signal strength" dictated by the avidity and affinity of the BCR-antigen interaction. However, a biochemical understanding of how these signals result in B cell non-responsiveness (anergy), rapid apoptosis (deletion) or continued Ig rearrangement (receptor editing) is a subject of continuing investigation.

Chronic exposure to antigen results in BCR desensitization, which is associated with impaired proximal signaling via Syk and Src family kinases, as well as the activation of some, but not all, downstream pathways (Cambier et al., 2007; Jun and Goodnow, 2003). Similarly, ablation of genes encoding negative or positive regulators of BCR signaling affect threshold-based negative (and positive) selection of emergent B cells (Cornall et al., 1998; Cyster et al., 1996). In addition to alterations in intracellular signaling, it is apparent that chronic engagement of the BCR also causes a redistribution of the BCR complex, first noted by the selective downregulation of surface IgM and, more recently, by evidence for disengagement of Ig-α-Ig-β signaling moieties, as well as a redistribution of BCR components in membrane microdomains (Goodnow et al., 1988; Vilen et al., 1999; Weintraub et al., 2000). Importantly, anergy is a reversible process and thus, mechanisms need to be in place to sustain the anergic state (Gauld et al., 2005; Goodnow et al., 1991). Anergic B cells exhibit low basal Ca^{2+} oscillations sufficient to activate calcineurin-dependent nuclear factor of activated T cells (NF-AT), but are refractory to induced Ca^{2+} flux (Healy et al., 1997). ERK activity is also elevated in the basal state, but is poorly induced in anergic B cells (Healy et al., 1997). Autoantibody production by anergic B cells may be prevented by sustained ERK signaling because this exerts an inhibitory effect on CpG-TLR9- and LPS-TLR4-dependent plasma cell differentiation (Rui et al., 2006; Rui et al., 2003). Activation of JNK and classical NF-κB are also impaired in anergic B cells, but experimental evidence is lacking as to the causative relationship of these pathways to the anergic state. Interestingly, PKCδ has emerged as an important regulator of B cell tolerance through the integration of signals via the BCR as well as BAFF-R (Mecklenbrauker et al., 2004; Mecklenbrauker et al., 2002). In particular, nuclear translocation of PKCδ appears to be a critical regulatory event in promoting apoptosis.

In recent years, PI3K signaling has moved to the forefront as a central pathway affecting peripheral B cell maturation. Class IA PI3K molecules are composed of regulatory (p85α, p55α, p50α, and p85β) and catalytic (p110α, p110β, and p110δ) subunits and phosphorylate PI(4,5)P_2 to generate the potent yet transient secondary messenger PI(3,4,5)P_3. Gene-targeting studies show similar defects in follicular, B-1, and marginal zone B cell subsets in *Pik3cd*$^{-/-}$ and *Pik3r1*$^{-/-}$ (encoding p110δ and p85α subunits, respectively) animals, indicating that the p85α-p110δ heterodimer is the most crucial form in B cells (Fruman and Bismuth, 2009). Recruitment of PI3K to the BCR

complex requires adaptor proteins bearing YXXM motifs that, upon tyrosine phosphorylation, can bind the SH2 domain of p85. Although cytosolic proteins such as the BCAP adaptors may participate, the transmembrane adaptor CD19 appears to be most crucial in recruiting PI3K (Aiba et al., 2007). In addition to serving as the signal-transducing component of the C3d-binding complement receptor CD21 (Rickert, 2005), CD19 interacts via noncovalent interactions with components of the BCR complex and thus is rapidly phosphorylated upon BCR cross-linking (Carter et al., 1997). Accordingly, B cell defects observed in CD19-deficient mice are similar in scope to that observed in *Pik3r1*$^{-/-}$ and *Pik3cd*$^{-/-}$ mice (Fruman and Bismuth, 2009).

PI3K activity is directly antagonized by the D3 inositol phosphatase and tumor suppressor PTEN that, although subject to regulation, is generally highly active and present at the plasma membrane in both resting and activated cells (Tamguney and Stokoe, 2007). Mice lacking PTEN in B cells possess enlarged marginal zone and B-1 cell compartments and present a "hyper-IgM" phenotype because of enhanced plasma cell differentiation and repressed class switch recombination (Anzelon et al., 2003; Omori et al., 2006; Suzuki et al., 2003). Conversely, most defects in peripheral B cell differentiation in mice lacking the PI3K adaptor CD19 are reverted by the dual loss of PTEN (Anzelon et al., 2003). Thus, the magnitude and duration of PI(3,4,5)P_3-dependent signaling is tightly regulated to govern B cell growth, survival, and differentiation.

We sought to determine whether the PI3K pathway modifies tolerogenic signaling in B cells. Using the hen egg lysozyme (HEL) neo-self antigen mouse model, we found that PI(3,4,5)P_3 was poorly generated in anergic B cells, resulting in reduced Akt activation. This deficit is due at least in part to impaired tyrosine phosphorylation of CD19 and elevated expression of PTEN. Importantly, PI(3,4,5)P_3 repression directly contributes to tolerogenic signaling, given that deletion of *Pten* results in failed B cell anergy and enhanced responsiveness to BCR signaling in newly formed B cells. These findings document the importance of the PI3K pathway in B cell tolerance, which may interface with other BCR-independent signaling pathways to govern the tolerogenic state.

RESULTS

Reduced PI(3,4,5)P_3 Induction in Anergic B Cells

Anergic B cells exhibit reduced activation of some BCR signaling pathways. We hypothesized that the restriction of PI(3,4,5)P_3 generation may also be a key component of the anergic state. To investigate this possibility, we obtained naive splenic B cells from MD4 transgenic mice that expressed an HEL-specific BCR and compared them with anergic B cells obtained from MD4ML5 mice, which express the MD4 Ig transgene, as well as the ML5 transgene that encodes soluble HEL. Splenocytes from each group were stimulated with anti-IgM F(ab')$_2$, fixed, and permeabilized and PI(3,4,5)P_3 amounts were assessed in B cells (B220$^+$) by intracellular staining with PI(3,4,5)P_3-specific antibodies. Upon stimulation, the amount of PI(3,4,5)P_3 increased in MD4 B cells, but not in MD4ML5 B cells (Figure 1A). The scarcity of PI(3,4,5)P_3 would prevent the PH domain-dependent docking and activation of Akt and other PH domain-bearing effectors. To determine whether this is the case in MD4ML5 cells, we stimulated MD4 and MD4ML5 B cells and assessed Akt phosphorylation (S473) by immunoblotting. Akt phosphorylation was induced in MD4 but not in MD4ML5 B cells upon stimulation with anti-IgM F(ab')$_2$ (Figure 1B, upper panels). Consistent with previous findings (Healy et al., 1997), Erk activation was basally elevated in MD4ML5 B cells and induced in both MD4 and MD4ML5 B cells (Figure 1B, upper panels). Because surface IgM is selectively downmodulated in MD4ML5 B cells, stimulations were also performed with HEL antigen or anti-Igκ to engage both IgM and IgD receptors. Measurements of Ca^{2+} flux or Akt (S473) phosphorylation was measured and found to be consistent with the anti-IgM F(ab')$_2$ data (Figure S1 available online). It is possible that dampened PI(3,4,5)P_3 signaling in splenic MD4ML5 B cells reflects the absence of marginal zone (MZ) B cells, which appear to be hyper-responsive to BCR signaling and are absent in MD4ML5 but present in MD4 mice (Mason et al., 1992; Oliver et al., 1997). Accordingly, we found reduced BCR-induced Akt activation in MD4 splenic B cells depleted of MZ B cells (Figure S2). However, Akt activation was impaired in MD4ML5 B cells relative to the follicular B cells from MD4 mice (Figure S2). Altogether, these findings indicate that PI3K activity is inhibited and/or inositol phosphatase activity is enhanced in anergic B cells.

CD19 Function in B Cell Anergy

Because the phosphorylation of CD19 upon BCR stimulation leads to the recruitment and activation of PI3K, we assessed whether signaling through CD19 was intact in anergic B cells. MD4 and MD4ML5 B cells were stimulated with anti-IgM F(ab')$_2$, and cell lysates were immunoblotted with antisera specific for one of the phosphorylated tyrosine residues (Y513) required for p85 binding to CD19. CD19 phosphorylation was induced in MD4 B cells, but not in MD4ML5 B cells (Figure 1B, lower panels). Surface expression of CD19 was unaltered in MD4ML5 cells (data not shown). This result suggests that in anergic B cells, Src kinase activity or functional association of CD19 with the BCR complex may be impaired.

To determine whether impaired CD19 signaling was a cause or a consequence of anergy, we crossed MD4 and MD4ML5 mice onto the *Cd19*$^{-/-}$ background. It has been reported that CD19 promotes positive selection of newly formed Ig-positive B cells (Buhl et al., 2000; Diamant et al., 2005); however, the role of CD19 modulation of BCR signaling in mediating negative selection has not been addressed. Consistent with previous findings (Buhl et al., 2000; Diamant et al., 2005), the loss of CD19 in MD4 B cells resulted in a modest reduction in peripheral B cell numbers (Figure 1C). Assessment of HEL-specific antibody revealed that tolerance mechanisms to prevent autoantibody production were intact in the absence of CD19 (Figure 1D). Both MD4ML5 and MD4ML5 *Cd19*$^{-/-}$ B cells selectively downregulated IgM (Figure 1E) and were short-lived, resulting in an equally reduced splenic B cell compartment relative to their naive counterparts (Figure 1C). Thus, altered BCR signaling thresholds in the absence of CD19 do not affect anergy induction.

PTEN Function in B Cell Anergy

PI(3,4,5)P_3 is rapidly hydrolyzed to PI(4,5)P_2 by the lipid phosphatase PTEN, which we hypothesize is another means by which PI(3,4,5)P_3 availability is regulated in autoreactive B cells.

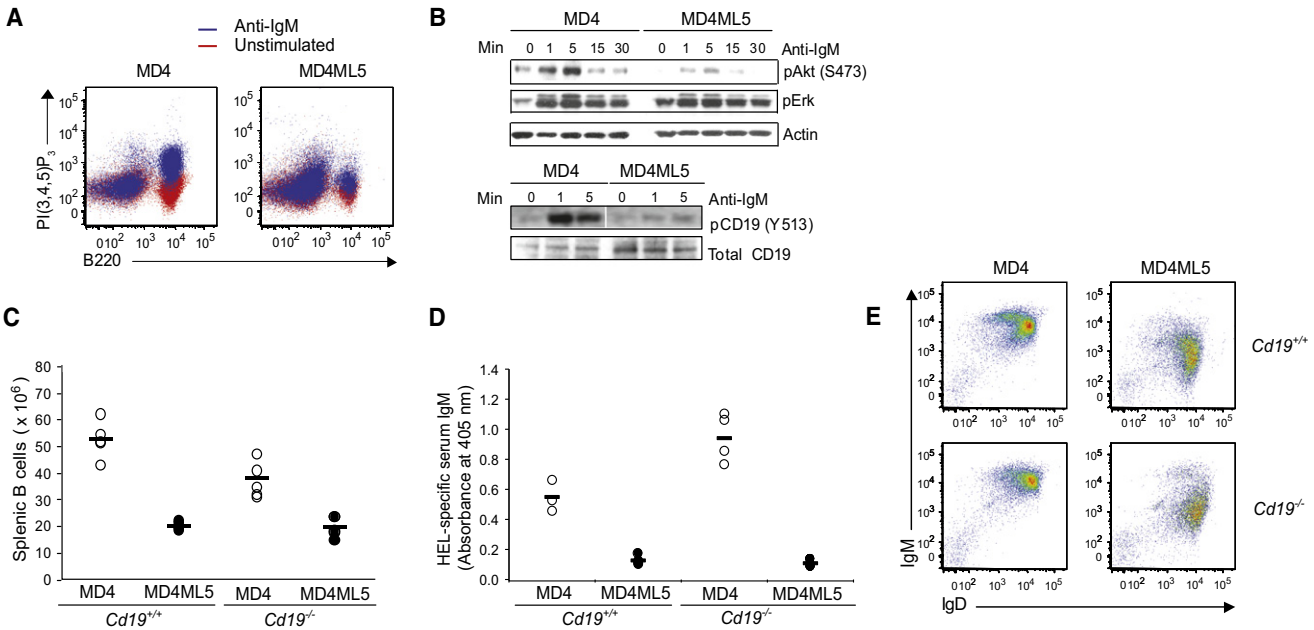

Figure 1. PI3kinase Signaling and CD19 Activation Are Inhibited in Anergic B Cells

(A) Splenic cells from MD4 (left panel) and MD4ML5 (right panel) mice were stimulated with anti-IgM F(ab')$_2$ (blue) or left unstimulated (red) for 5 min. Cells were fixed, permeabilized, stained with antibodies against B220 and PI(3,4,5)P_3, and analyzed by flow cytometry.

(B) As shown in the upper panel, purified MD4 and MD4ML5 B cells were stimulated with anti-IgM F(ab')$_2$ for the indicated times. Cell lysates were immunoblotted with anti-phosphorylated Akt (S473), anti-phosphorylated Erk, and anti-actin. As shown in the lower panels, purified MD4 and MD4ML5 B cells were stimulated with anti-IgM F(ab')$_2$ for the indicated times. Cell lysates were immunoblotted with anti-phosphorylated CD19 (Y513) and anti-CD19.

(C) Splenic B cells of MD4-*Cd19*$^{+/+}$, MD4ML5-*Cd19*$^{+/+}$, MD4-*Cd19*$^{-/-}$, and MD4ML5-*Cd19*$^{-/-}$ mice were enumerated.

(D) HEL-specific IgM titers from MD4-*Cd19*$^{+/+}$, MD4ML5-*Cd19*$^{+/+}$, MD4-*Cd19*$^{-/-}$, and MD4ML5-*Cd19*$^{-/-}$ mice were determined by ELISA.

(E) IgM and IgD profiles of B cells from MD4-*Cd19*$^{+/+}$, MD4ML5-*Cd19*$^{+/+}$, MD4-*Cd19*$^{-/-}$, and MD4ML5-*Cd19*$^{-/-}$ mice were determined by flow cytometry.

To assess PTEN protein expression in naive and anergic B cells, we prepared whole-cell lysates from MD4 and MD4ML5 B cells and immunoblotted them for PTEN expression. Interestingly, PTEN protein expression was found to be higher in MD4ML5 B cells compared to MD4 B cells (Figure 2A). Flow cytometric analysis revealed similar amounts of PTEN in the MZ and follicular B cell compartments of MD4 mice (data not shown). Given that modest changes in PTEN expression can have striking effects on cellular responses, these results suggest that PTEN may be required to suppress signaling via the PI3K pathway in autoreactive B cells.

Because PI(3,4,5)P_3 induction is reduced in anergic B cells, we postulated that inactivation of PTEN in maturing B cells would result in elevated PI(3,4,5)P_3 and the unabated activation of downstream effector molecules would cause a break in B cell tolerance. To test this hypothesis, we crossed MD4 mice and MD4ML5 mice with *Pten*$^{flox/flox}$-*Cd19*-Cre mice to inactivate *Pten* specifically in B cells and to allow for the analysis of B cell anergy induction and maintenance. The MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ animals used were all heterozygous for *Cd19* and express Cre recombinase. BCR-induced PI(3,4,5)P_3 generation was measured in splenic cells obtained from MD4-*Pten*$^{flox/flox}$ and MD4ML5-*Pten*$^{flox/flox}$ mice. Splenic cells were stimulated with anti-IgM F(ab')$_2$, intracellularly stained with anti-PI(3,4,5)P_3, and analyzed by flow cytometry (Figure 2B). In contrast to the inhibition of PI(3,4,5)P_3 induction seen in MD4ML5 B cells (Figure 1A), MD4ML5-*Pten*$^{flox/flox}$ B cells showed an increase in PI(3,4,5)P_3 upon stimulation (Figure 2B). Downstream signaling was assessed by measuring Ca^{2+} flux in MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ B cells stimulated with anti-IgM F(ab')$_2$ or HEL antigen. As expected, MD4 B cells mobilized Ca^{2+} efficiently when stimulated with either anti-IgM F(ab')$_2$ or HEL, whereas MD4ML5 B cells did not (Figure 2C, left panels). In contrast, MD4-*Pten*$^{flox/flox}$ and MD4ML5-*Pten*$^{flox/flox}$ B cells were both able to mobilize Ca^{2+} (Figure 2C, right panels), indicating the continued ability to induce productive BCR signaling. To further confirm the restoration of BCR signaling in MD4ML5-*Pten*$^{flox/flox}$ B cells, we prepared lysates from anti-IgM- or anti-Igκ stimulated-MD4ML5 and MD4ML5-*Pten*$^{flox/flox}$ B cells and immunoblotted them for activated Akt (Figure 2D and Figure S3). Whereas Akt was not activated in MD4ML5 B cells, MD4ML5-*Pten*$^{flox/flox}$ B cells efficiently activated Akt (Figure 2D and Figure S3). Erk was basally phosphorylated in MD4ML5 but not MD4ML5-*Pten*$^{flox/flox}$ B cells, although it was inducible in both B cell types (Figure 2D, lower panels). These results suggest that the loss of *Pten* prevents B cells from acquiring a biochemical profile consistent with the anergic state.

The hallmarks of B cell anergy include the selective downregulation of surface IgM, as well as limited cell lifespan and proliferation and an inability to become immunoglobulin-secreting cells. Therefore, we sought to determine whether the observed recovery of productive BCR signaling by autoreactive PTEN-deficient B cells translated into increased B cell numbers in the

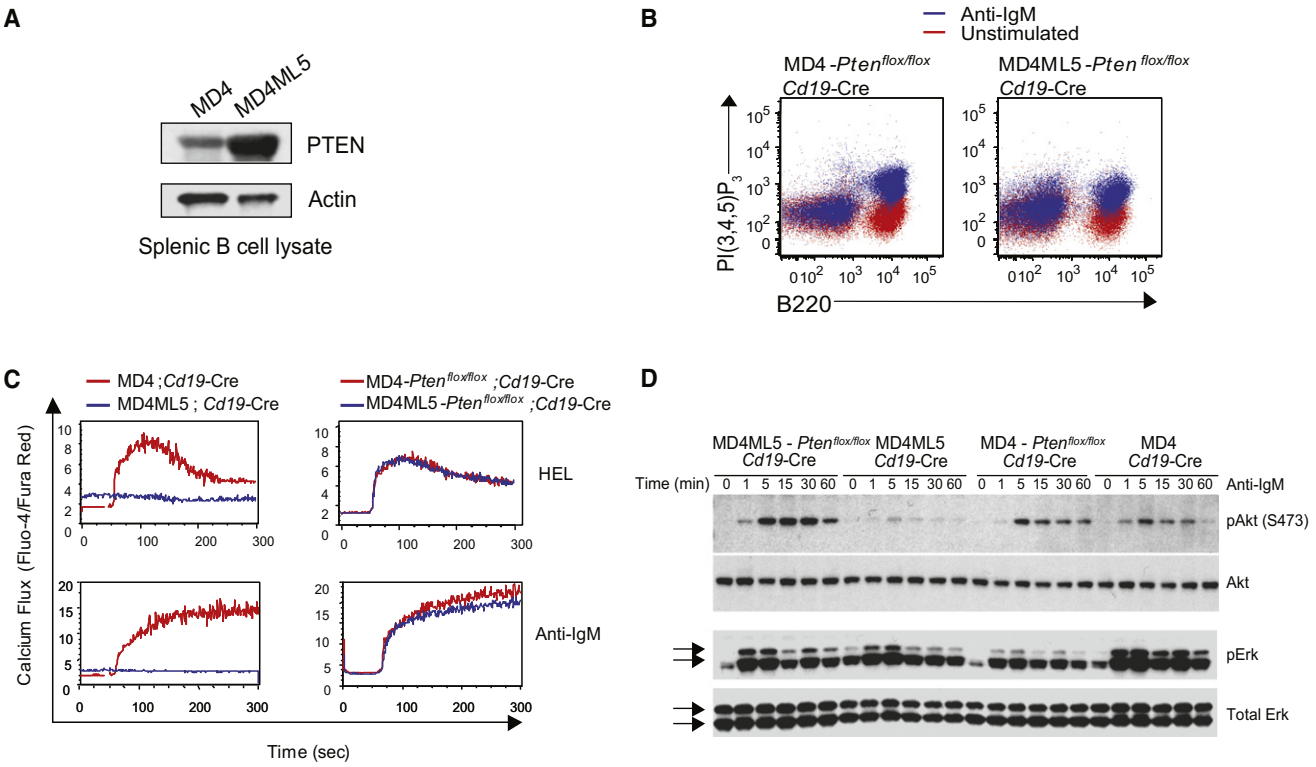

Figure 2. PTEN Protein Expression Is Increased in Anergic B Cells and PI3 Kinase Signaling Is Recovered in PTEN-Deficient B Cells

(A) Purified B cells from MD4 and MD4ML5 mice were lysed and immunoblotted with anti-PTEN or anti-actin.

(B) Splenic cells from MD4- *Pten*$^{flox/flox}$ and MD4ML5-*Pten*$^{flox/flox}$ mice were stimulated with anti-IgM F(ab')$_2$ (blue) or left unstimulated (red) for 5 min. Cells were fixed, permeabilized, stained with antibodies against B220 and PI(3,4,5)P_3, and analyzed by flow cytometry.

(C) Purified splenic B cells from MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were stimulated with 10 μg/ml HEL (upper panels) or 10 μg/ml anti-IgM F(ab')$_2$ (lower panels), and Ca^{2+} flux was measured by Fluo-4(530 nm)/Fura Red(685 nm) emission ratiometry. MD4 and MD4-*Pten*$^{flox/flox}$ cells are depicted in red, whereas MD4ML5 and MD4ML5-*Pten*$^{flox/flox}$ cells are depicted in blue.

(D) Purified MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ splenic B cells were stimulated with anti-IgM F(ab')$_2$ for the indicated times, and cell lysates were immunoblotted with antibodies against phosphorylated Akt (S473), total Akt, phosphorylated Erk, and total Erk.

periphery and increased titers of serum autoantibody. Although MD4ML5 mice had low splenic and lymph node B cell numbers compared to their naive counterparts, the B cell compartment in MD4ML5-*Pten*$^{flox/flox}$ mice was comparable to their naive counterparts (Figure 3A and Figure S4). Interestingly, the MZ B cell compartment is restored in MD4ML5-*Pten*$^{flox/flox}$ mice, but is not expanded to the extent observed in MD4-*Pten*$^{flox/flox}$ mice (Figure S5). The ability of B cells to secrete immunoglobulin was assessed by measuring HEL-specific serum IgM titers by ELISA. We found that whereas MD4 mice had substantially higher serum IgM titers than MD4ML5 mice, MD4-*Pten*$^{flox/flox}$ mice and MD4ML5-*Pten*$^{flox/flox}$ mice both maintained high titers of serum IgM (Figure 3B). Furthermore, whereas MD4ML5 B cells dramatically downregulated surface IgM, MD4ML5-*Pten*$^{flox/flox}$ B cells did so to a lesser extent (Figure 3C). These results demonstrate that the loss of PTEN leads to failed anergy and the production of abundant autoantibody.

Effects of PTEN Loss on Self-Antigen Availability and Receptor Occupancy

Secretion of autoantibody or the expression of autoreactive B cell receptors can effectively sequester self-antigen and impact B cell tolerance, given that continued B cell receptor occupancy is necessary for the maintenance of tolerance (Gauld et al., 2005; Goodnow et al., 1989). Receptor occupancy, as defined by the amount of HEL bound to the surface of B cells, was measured in MD4, MD4ML5, and MD4ML5-*Pten*$^{flox/flox}$ B cells by flow cytometry with a polyclonal HEL antibody. We found that freshly isolated MD4ML5-*Pten*$^{flox/flox}$ B cells had ~10-fold lower bound HEL than MD4ML5 B cells (Figure 4A). This finding suggests that receptor occupancy in vivo is reduced on autoreactive *Pten*-deficient B cells, which may contribute to the inability to maintain tolerance. Low receptor occupancy resulting in the increased presence of autoreactive B cells may be more prominent in adult mice as serum IgM titers accumulate and deplete available HEL. To address this possibility, we quantified receptor occupancy in adult (>8 weeks old) and young (3 weeks old) mice by incubating splenic B cells with excess exogenous HEL to fully occupy surface receptors. The amount of surface HEL on these cells was compared to the amount of surface HEL on cells treated with PBS alone; hence, the degree of receptor occupancy was determined as the ratio of the mean fluorescence intensities (MFI) of the PBS- and HEL-treated samples. In adult MD4ML5 mice, the amount of surface HEL on PBS-treated B cells was

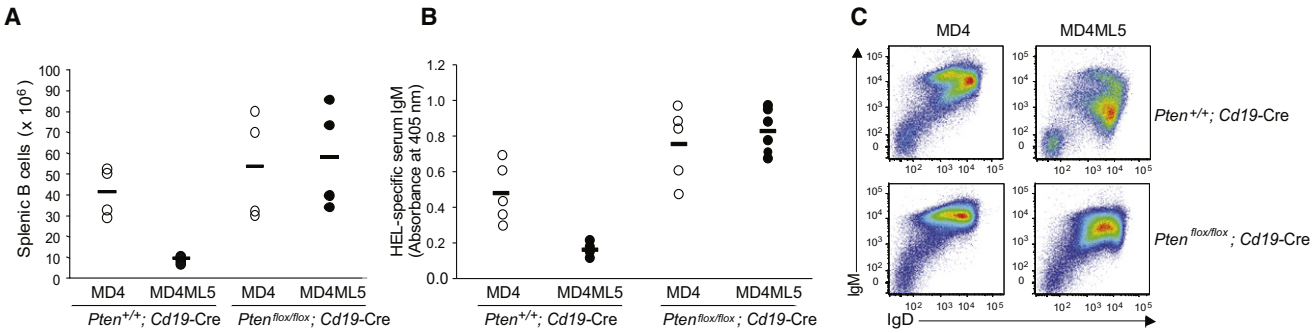

Figure 3. MD4ML5 PTEN-Deficient Mice Do Not Display an Anergic Phenotype

(A) Splenic B cells from MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were enumerated.

(B) HEL-specific serum IgM titers from MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were determined by ELISA.

(C) IgM and IgD profiles of B cells from MD4, MD4ML5, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were determined by flow cytometry.

50% that of HEL-treated B cells (Figure 4B), which has been previously reported as sufficient MD4 receptor occupancy by endogenous antigen to induce and maintain tolerance (Goodnow et al., 1989). Consistent with the hypothesized sequestration of HEL by secreted or membrane-bound IgM in adult MD4ML5-*Pten*$^{flox/flox}$ mice, only 10% of MD4 receptors were occupied by endogenous HEL (Figure 4B). Interestingly, in young mice, receptor occupancy on both MD4ML5 and MD4ML5-*Pten*$^{flox/flox}$ B cells was approximately equivalent (~60%) (Figure 4B), suggesting a cumulative effect of self-antigen sequestration in MD4ML5-*Pten*$^{flox/flox}$ mice.

To qualitatively assess HEL antigen expression in MD4ML5-*Pten*$^{flox/flox}$ mice, we introduced CFSE-labeled MD4 B cells into MD4ML5 and MD4ML5-*Pten*$^{flox/flox}$ mice by tail-vein injection and measured the downregulation of surface IgM upon acute in vivo antigen encounter. We found that donor MD4 B cells downregulated surface IgM strongly when exposed for 24 hr to the adult MD4ML5 environment, suggesting a productive encounter with HEL antigen (Figure 4C). In contrast, when donor MD4 B cells were exposed to the adult MD4ML5-*Pten*$^{flox/flox}$ environment, surface IgM expression was downregulated to a lesser extent (Figure 4C). In both cases, the downregulation of surface IgM was antigen dependent because donor MD4 B cells transferred into non-HEL-expressing MD4 and MD4-*Pten*$^{flox/flox}$ environments maintained similarly high basal expression of surface IgM (Figure 4C). Thus, less HEL is available in adult MD4ML5-*Pten*$^{flox/flox}$ animals for inducing and sustaining anergy.

As previously reported (Schmidt and Cyster, 1999), acute encounter with HEL leads to the elimination of antigen-specific MD4 B cells in vivo. MD4, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were administered exogenous HEL or PBS, and splenocytes were harvested 48 hr later for assessment of splenic B cell numbers, surface IgM downregulation, and upregulation of CD86. The introduction of exogenous HEL induced a measurable decrease in surface IgM in all groups, whereas CD86 was only upregulated on MD4-*Pten*$^{flox/flox}$ B cells (data not shown). Importantly, MD4-*Pten*$^{flox/flox}$ and MD4ML5-*Pten*$^{flox/flox}$ B cells expanded or persisted in the presence of additional HEL, respectively (Figure 4D), whereas MD4 B cells were eliminated. These findings indicate that increasing the concentration of free self-antigen confers an anergic phenotype on MD4ML5-*Pten*$^{flox/flox}$ B cells, but they remain long-lived.

The frequency of autoreactive B cells in normal mice is relatively low, and the abundance of anti-HEL B cells in the MD4ML5 system affects the concentration of available HEL self-antigen. Therefore, bone marrow chimeras were generated for assessing whether the ability of PTEN-deficient, self-reactive B cells to escape the induction of anergy was independent of their frequency within the B cell repertoire. Lethally irradiated ML5 mice were reconstituted with a donor population of lineage-depleted hematopoietic stems cells composed of a 20:80 mixture of either nonTg:MD4 or nonTg:MD4-*Pten*$^{flox/flox}$ cells. After 12 weeks of reconstitution, we found that despite similar seeding frequencies, MD4-*Pten*$^{flox/flox}$ B cells survived and produced abundant autoantibody, whereas MD4 B cells were present at a lower frequency and produced little autoantibody (Figure 5), consistent with previous findings (Cyster et al., 1994). These results confirm that PTEN deficiency in self-reactive B cells impairs the induction or maintenance of the anergic state.

Immature PTEN-Deficient B Cells Are Less Sensitive to Tolerogenic Signals

Negative selection takes place at the immature B cell stage during which engagement of the B cell receptor with self-antigen leads to B cell anergy or death. One of the cellular strategies to induce anergy is through intrinsic negative regulation of key signaling components. To determine the contribution of PI(3,4,5)P_3 signaling to negative selection of newly formed B cells expressing a normal diverse repertoire of B cell receptors, we measured the activation and proliferation in response to BCR engagement of cultured immature B cells from nontransgenic wild-type and *Pten*$^{flox/flox}$ mice. Bone marrow from nontransgenic wild-type and *Pten*$^{flox/flox}$ mice were harvested and cultured with IL-7 over a 6 day period for promoting the selective expansion of pre-B cells. Cells were then labeled with CFSE and either returned to IL-7 or removed from IL-7 so that they could transition to immature IgM-expressing B cells. In the latter culture, B cells were treated with anti-IgM F(ab')$_2$ fragments (1 or 10 μg/ml) to engage the BCR on newly formed IgM-positive cells. B cells (B220$^+$) were enumerated and examined for an activated profile (CD86$^+$, increased forward scatter) as well as proliferative capacity (CFSE partitioning) over a 3 day period. CD86 staining showed a ~7-fold enhancement in the frequency of

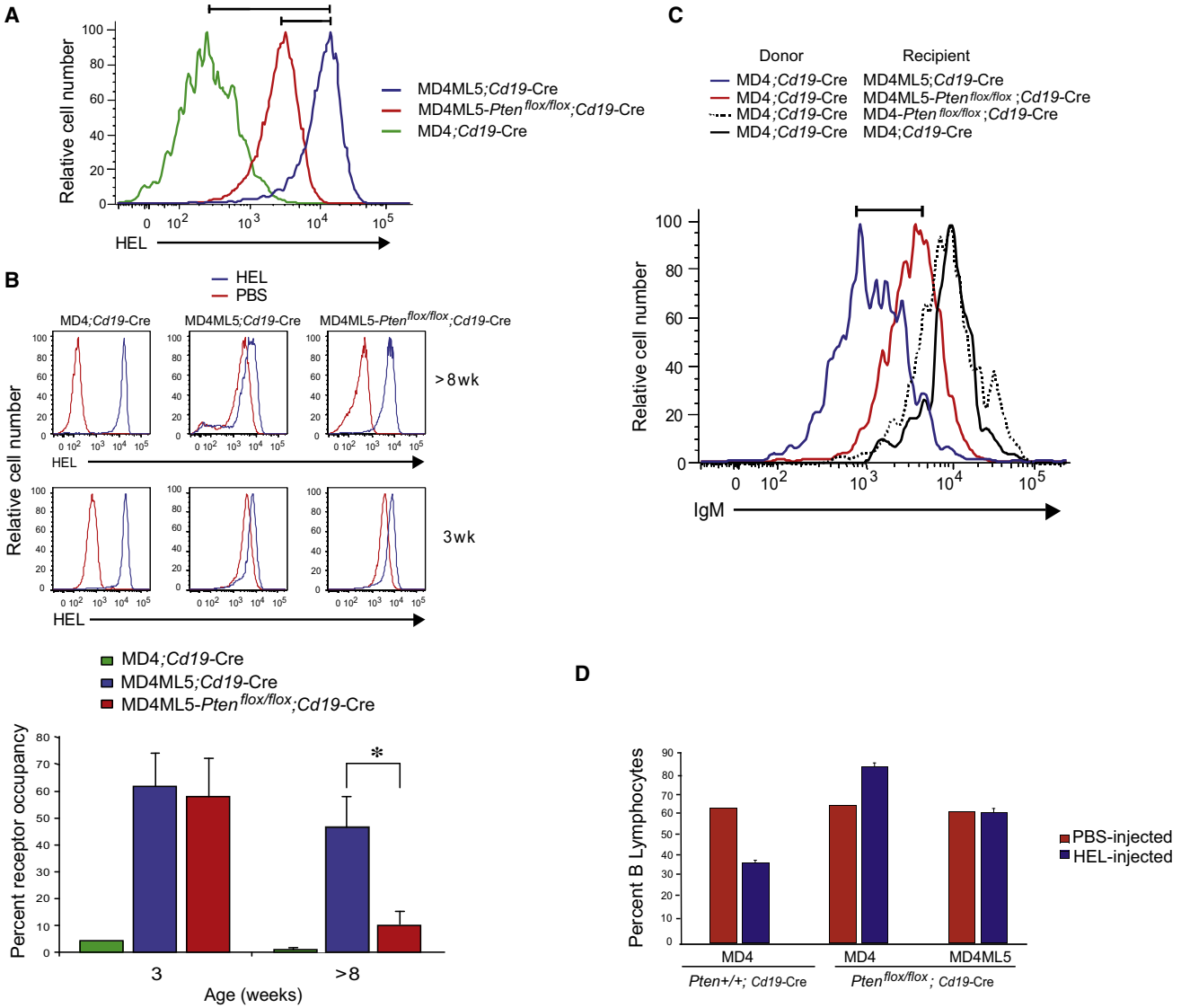

Figure 4. MD4ML5 PTEN-Deficient B Cells Exhibit Low Receptor Occupancy

(A) Surface HEL staining of MD4 (green), MD4ML5 (blue), and MD4ML5-*Pten*$^{flox/flox}$ (red) B cells was determined by flow cytometry.

(B) MD4, MD4ML5, and MD4ML5-*Pten*$^{flox/flox}$ splenic cells from adult mice (>8 weeks old; upper panels) and young mice (3 weeks old; lower panels) were incubated in 20 μg/ml HEL (blue) or PBS (red). Surface HEL was detected with polyclonal antibody against HEL and measured by flow cytometry. As shown in the bar graph (bottom), percent receptor occupancy was determined from the ratio of HEL staining intensity of PBS-incubated cells to HEL-incubated cells. The asterisk indicates a p value of < 0.05.

(C) MD4 B cells were CFSE-labeled and equal cell numbers were transferred into MD4ML5 (blue), MD4ML5-*Pten*$^{flox/flox}$ (red), MD4-*Pten*$^{flox/flox}$ (dotted), and MD4 (black) recipient mice by tail-vein injection. After 24 hr, recipient splenic cells were harvested and stained with anti-B220 and anti-IgM. CFSE+ donor B cells were gated, and surface IgM was analyzed by flow cytometry.

(D) MD4, MD4-*Pten*$^{flox/flox}$, and MD4ML5-*Pten*$^{flox/flox}$ mice were injected with HEL (blue) with three mice per group or PBS (red) with one mouse per group. Splenic cells were isolated at 48 hr postinjection, enumerated, and examined for expression of B220 by flow cytometry. Histograms represent B220-positive cells.

activated immature *Pten*$^{flox/flox}$ B cells relative to immature wild-type B cells (Figure 6A). Gating on activated B cells revealed that immature *Pten*$^{flox/flox}$ B cells proliferated to a much greater extent than immature wild-type B cells (Figure 6B). These findings suggest that sustained PI(3,4,5)P_3 signaling converts what is normally a tolerogenic response into a mitogenic response during B cell development.

Neonatal B cells exhibit phenotypic and functional characteristics similar to that of immature B cells of adult bone marrow. To assess the effects of PI(3,4,5)P_3 signaling on tolerance induction during this developmental stage, we analyzed 5-day-old neonatal littermates consisting of nontransgenic wild-type and nontransgenic *Pten*$^{flox/flox}$ mice. Interestingly, neonatal spleens were not markedly enlarged in *Pten*$^{flox/flox}$ mice as we typically see in adult *Pten*$^{flox/flox}$ mice (Anzelon et al., 2003). Moreover, surface IgM and IgD profiles of wild-type and *Pten*$^{flox/flox}$ B cells from neonatal spleens revealed similarly high frequencies of IgM$^+$IgD$^-$ B cells compared to adult B cells, which are largely

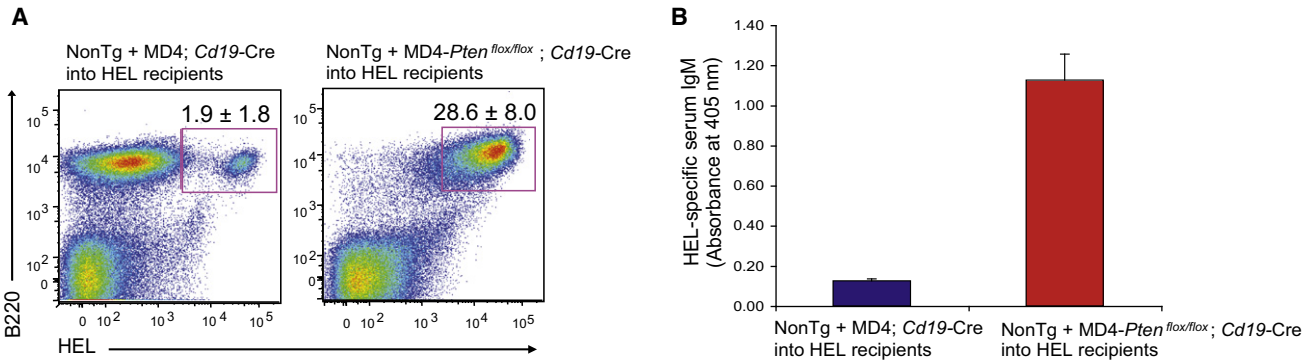

Figure 5. MD4ML5 PTEN-Deficient B Cells Evade Anergy in Mixed Bone Marrow Chimeras

(A) Reconstitution with mixed bone marrow HSCs consisting of Non-Tg wild-type (20%) plus either MD4 (80%, left panel) or MD4-*Pten*$^{flox/flox}$ (80%, right panel) in ML5 recipients (n = 3 for each group). The frequencies of HEL-specific B cells were determined after 12 weeks of reconstitution.

(B) Sera from bone marrow chimeric recipient animals were analyzed for HEL-specific IgM by ELISA. Absorbances are depicted in the bar graph. Each bar represents the average of three chimeric mice.

IgM$^+$IgD$^+$ (Figure 6C, left panels). Using established protocols (Chang et al., 1991; Yellen et al., 1991), we harvested and cultured B cells from neonatal and adult wild-type and *Pten*$^{flox/flox}$ mice in media containing IL-4 alone, IL-4 plus LPS, or IL-4 plus anti-IgM F(ab')$_2$. Proliferation was assessed by ^{3}H-thymidine incorporation (Figure 6C, right panel). Wild-type neonatal B cells proliferated modestly in response to LPS, but no proliferation was observed in response to anti-IgM F(ab')$_2$, confirming that BCR stimulation is inhibitory in wild-type neonatal B cells (Figure 6C). In contrast, neonatal *Pten*$^{flox/flox}$ B cells proliferated strongly in response to both LPS and anti-IgM F(ab')$_2$ (Figure 6C). These findings corroborate the results of the bone marrow culture system (Figures 6A and 6B) and demonstrate that sustained PI(3,4,5)P_3 signaling leads to activation and proliferation rather than inhibition and anergy in immature B cells upon BCR engagement.

DISCUSSION

Despite the striking differences in responsiveness to antigen by immature and mature B cells, the underlying biochemical bases for these distinct responses are unclear. Because PI3K signaling is central to cellular growth control, proliferation, and survival, the current work focused on the role of the PI3K pathway in the induction and maintenance of B cell anergy. In mature B cells, BCR engagement leads to the rapid generation of PI(3,4,5)P_3 as a consequence of PI3K (p85 and p110) recruitment to CD19 and membrane-proximal adaptor proteins. In anergic B cells, we found that PI(3,4,5)P_3 production was substantially reduced. Consistent with this observation, CD19 was expressed to a normal degree, but was not efficiently phosphorylated on the tyrosine residues that mediate PI3K recruitment. This defect may be a consequence of impaired tyrosine kinase activity, as has been noted in anergic B cells (Cooke et al., 1994). Alternatively, CD19 may be physically uncoupled from the BCR complex upon chronic engagement of the BCR; such a mechanism has been described for the redistribution of the Ig-α-Ig-β heterodimer (Vilen et al., 1999), and recent data indicate that CD19 function is crucial in the formation of and signaling by antigen-bound BCR microclusters (Depoil et al., 2008). Interestingly, overexpression of human CD19 has been shown to cause a break in tolerance (Inaoki et al., 1997), probably resulting from a preferential association of human CD19 with the BCR (given that it interacts inefficiently with murine CD21 [Hasegawa et al., 2001]). Coengagement of the BCR and CD21-CD19 Complement Receptor 2 complex by C3d-bearing self-antigens can also overcome peripheral tolerance (Del Nagro et al., 2005; Lyubchenko et al., 2007), suggesting that forced recruitment of CD19 into the BCR complex overcomes receptor desensitization to augment PI3K activation.

Given these findings, it is perhaps not surprising that anergy was not affected by the loss of CD19. By contrast, the loss of CD19 appears to impair positive selection of newly formed B cells in both immunoglobulin transgenic as well as nontransgenic *Cd19*$^{-/-}$ mice (Buhl et al., 2000; Diamant et al., 2005). We have confirmed that this is the case for the HEL system as *Cd19*$^{-/-}$ MD4 mice have reduced peripheral B cells relative to wild-type MD4 counterparts (Figure 1C). These findings suggest that BCR-associated CD19 is critical for the propagation of weak signals during positive selection, but is dispensable for negative selection mediated by strong signals (such as binding of HEL by the MD4 receptor). Consistent with this view are our previous findings of reduced survival of follicular CD19-deficient B cells responding to submitogenic "tonic" signaling but intact antigen-driven responses to potent multivalent antigens (Otero et al., 2003; Rickert et al., 1995).

In addition to impaired CD19 signaling, we found elevated expression of PTEN in anergic B cells. Whereas loss of PTEN expression is oncogenic in most cells, increased PTEN expression or stability can further suppress growth factor receptor signaling (Tamguney and Stokoe, 2007). Given that PTEN loss leads to failed anergy, we conclude that reduced PI(3,4,5)P_3 is not only a novel hallmark of anergic B cells but is also a prerequisite for anergy induction and maintenance. Relatedly, PI3K has also been recently shown to negatively regulate receptor editing by modulating RAG expression (Verkoczy et al., 2007). PI(3,4,5)P_3 is transiently induced upon cell activation and triggers multiple downstream pathways via the recruitment of pivotal PH domain-containing proteins. In addition to the BCR, signaling via costimulatory receptors and accessory molecules is also

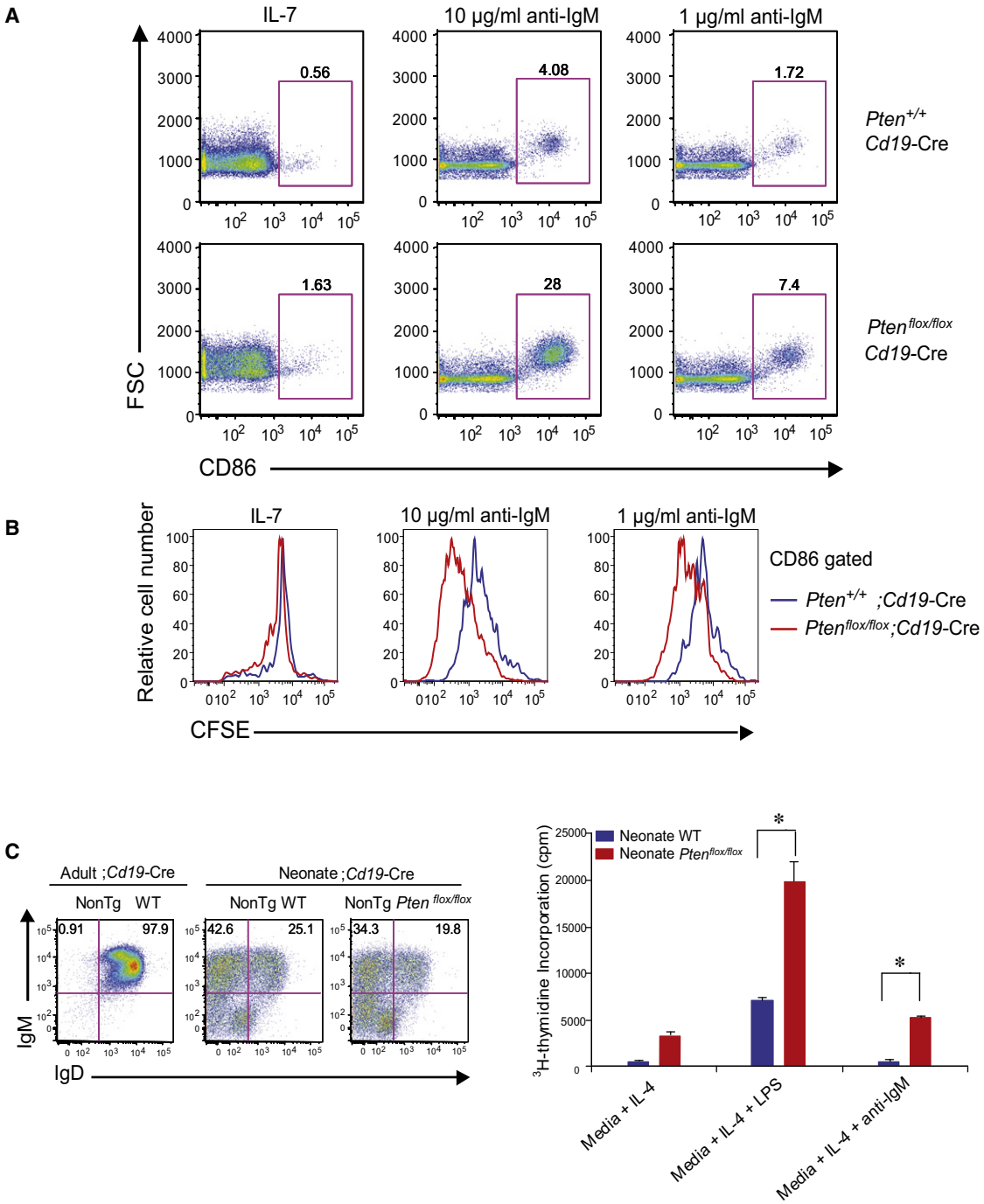

Figure 6. Negative Selection Is Impaired in MD4ML5 PTEN-Deficient Mice

(A) Nontransgenic wild-type and PTEN-deficient bone marrow B cells were purified, and cells were cultured in rIL-7 for 6 days. Cells were labeled with CFSE and cultured with 10 μg/ml or 1 μg/ml anti-IgM F(ab')$_2$ or with 10 ng/ml rIL-7 for 3 days. Cells were collected and stained for B220, 7AAD, and CD86. 7AAD-, B220+ cells were gated.

(B) CD86$^+$ cells above were analyzed for CFSE and the degree of proliferation was assessed in CD86-gated subsets.

(C) As shown in the left panel, IgM-IgD profiles of splenic B cells from adult wild-type, neonate wild-type, and neonate PTEN-deficient nontransgenic mice were analyzed by flow cytometry. As shown in the right panel, splenic B cells from a 5-day-old nontransgenic wild-type neonatal mouse (blue) and a 5-day-old nontransgenic PTEN-deficient neonatal mouse (red) were harvested and cultured in IL-4 alone or in IL-4 plus either LPS or anti-IgM F(ab)'$_2$ in a 96-well plate. After 48 hr in culture, 1 μCi of ^{3}H-thymidine was added to each well, and triplicate wells were harvested 16 hr later. Proliferation was measured by scintillation counting. This is a representative of two experiments. Asterisks indicate a p value of < 0.05.

augmented in the absence of PTEN (Anzelon et al., 2003; Suzuki et al., 2003). Of particular interest is threshold-based signaling via the BAFF-R, which utilizes the PI3K pathway and regulates the fate of anergic B cells in concert with the BCR (Lesley et al., 2004; Patke et al., 2006; Thien et al., 2004). Normal hyporesponsive PI3K signaling downstream of the BCR and BAFF-R in self-reactive B cells undergoing anergy could be overcome by a loss of PTEN, resulting in a breach in tolerance. This hypothesis predicts that haplo-insufficient or B cell-specific PTEN-deficient animals would be prone to autoantibody production and autoimmune disease. Indeed, although autoantibody production has been noted in *Pten* mutant mice (Di Cristofano et al., 1999; Suzuki et al., 2003), we postulate that autoantibody-associated disease is generally not observed because elevated PI3K signaling negatively regulates class switch recombination and, hence, the production of pathogenic IgG (Dengler et al., 2008; Omori et al., 2006).

The strength of signaling via the BCR is the key determinant of B cell fate after self-antigen encounter. Weak signals induced by low-affinity and/or -avidity interactions induce the anergic state, which can lead to apoptosis in the short term, but this fate can be averted upon removal of antigen, change in BCR specificity, or provision of costimulatory signals (Cooke et al., 1994; Cornall et al., 1998; Gauld et al., 2005; Halverson et al., 2004; Healy and Goodnow, 1998; Phan et al., 2003). In immunoglobulin transgenic systems, the affinity of the BCR for self-antigen is fixed; however, the strength of signal can be modulated by the relative amount or nature of the self-antigen (soluble versus membrane bound). Given that membrane or secreted anti-HEL immunoglobulin can deplete self-antigen, under some circumstances local HEL may become subthreshold, resulting in the accumulation of HEL-specific B cells that are "indifferent" or "ignorant" of self-antigen. Early studies using the soluble HEL system showed that receptor occupancy of ~50% was sufficient to induce and maintain anergy (Goodnow et al., 1989). This threshold correlates with the in vivo positioning of anergic B cells in the outer PALS (Cook et al., 1997; Cyster et al., 1994; Fulcher et al., 1996) and can be recapitulated with the transfer of naive immature or mature Ig transgenic B cells into HEL transgenic recipients (Fulcher et al., 1996). Our B cell transfer and HEL infusion experiments indicate that PTEN-deficient B cells in adult animals are exposed to lower amounts of HEL and thus are less likely to adopt the phenotypic and functional characteristics of anergy. Nonetheless, we also demonstrate using mixed bone marrow chimeras that PTEN loss causes an intrinsic defect in B cell signaling, resulting in impaired induction of maintenance of B cell anergy. These findings are consistent with our studies of nontransgenic PTEN-deficient immature B cells that were isolated from adult bone marrow or neonatal or adolescent mice and that were found to be responsive to BCR signaling, indicating a primary defect in tolerogenic signaling in newly formed B cells that lack PTEN. These findings establish PI(3,4,5)P_3 metabolism as a focal point for inducing and maintaining the tolerogenic state in B cells, setting the stage for future studies exploring the role of particular PI(3,4,5)P_3 effectors in this context.

In summary (Figure S6), we propose that elevated and sustained activation of the PI3K pathway in newly formed B cells results in altered negative selection and the egress of autoreactive B cells into the periphery. Once in the periphery, these cells also appear to be refractory to elimination by the continued presence of self-antigen. Interestingly, because elevated PI3K signaling favors MZ B cell formation (Anzelon et al., 2003; Suzuki et al., 2003), selection into this compartment may promote the propagation and further differentiation of autoreactive B cells in response to BAFF, TLR ligands, and T cell-derived factors, as others have suggested (Enzler et al., 2006; Thien et al., 2004). In addition to these intrinsic effects, the accumulation of autoreactive B cells and secreted autoantibody will gradually sequester HEL, leading to the release and maturation of additional "clonally ignorant" B cells as a secondary event. This effect is dramatically revealed with the monoclonal HEL system given that self-reactive B cells are present in great abundance (even in a mixed bone marrow chimera) and express or secrete high-affinity immunoglobulin. However, the chronicity of autoimmune disease and long-lived nature of antigen-selected B cells support the broad premise that self-antigen depletion by autoantibody may perpetuate autoimmunity through the continued release of self-reactive bone marrow B cells, some of which may persist and differentiate into autoantibody-producing cells in the periphery.

EXPERIMENTAL PROCEDURES

Mice

Mice expressing MD4 (HEL-Ig transgene) and ML5 (sHEL transgene) were obtained from Jackson Laboratories (Goodnow et al., 1988). These mice were bred so that double transgenic mice (MD4ML5) could be obtained. *Pten*$^{flox/flox}$ mice (Lesche et al., 2002) were crossed with CD19*cre* mice (in which cre recombinase expression is driven by the CD19 promoter [Rickert et al., 1997]) for *Pten*$^{flox/flox}$-*Cd19*-Cre mice generation. Single-transgenic (MD4) and double-transgenic (MD4ML5) mice were subsequently bred with *Pten*$^{flox/flox}$-*Cd19*-Cre mice for obtaining naive (MD4) and autoreactive (MD4ML5) mice with a B cell-specific *Pten* deletion. All animals were maintained in an animal facility, and experimental procedures were approved by the IACUC committee at The Burnham Institute for Medical Research (La Jolla, CA).

Immunoblotting

Splenic B cells were purified with MACS beads for negative selection of B cells, in accordance with the manufacturer's recommended procedure (Miltenyi Biotech). One to ten million B cells were washed with PBS and stimulated with 10 μg/mL goat anti-mouse IgM F(ab')$_2$ (Jackson ImmunoResearch Laboratories) for the indicated times at 37°C. Cell pellets were lysed on ice for 30 min in either RIPA lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.1% SDS) or NP-40 lysis buffer (20 mM Tris-HCl [pH 7.5], 1% NP-40, 10% glycerol, 10 mM NaCl, and 1 mM EDTA) plus protease inhibitors (2 μg/ml leupeptin, 2 mM PMSF, 2 μg/ml aprotinin, and 1 mM sodium orthovanadate). Lysates were electrophoresed with 4%–12% acrylamide SDS gels and blotted onto nitrocellulose paper. Antibodies against CD19, phospho-CD19, Akt, phospho-Akt, Erk, phospho-Erk, and actin were from Cell Signaling Technology. Proteins were revealed with HRP-labeled anti-rabbit antibodies and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Flow Cytometry

Spleens were excised and red blood cells depleted from cell suspensions with hypotonic ammonium chloride. One million splenic cells were resuspended in FACS buffer (PBS, 1% FBS, and 0.01% sodium azide) and incubated with the following conjugated antibodies: IgM-APC, IgD-PE, B220-APC-Cy7, and CD11b-PE-Cy7 (BD Bioscience or eBioscience). Samples were washed with FACS buffer and analyzed with a FACSCanto flow cytometer and FlowJo software (Treestar). For PI(3,4,5)P_3 staining, 5 × 10^6 splenic cells were

resuspended in 100–200 μl PBS, preincubated at 37°C for 5 min, and stimulated with anti-IgM F(ab')$_2$ at a final concentration of 10 μg/ml at 37°C for 5 min. Cells were fixed with 1.5% formaldehyde at RT for 10 min, washed with cold PBS, and resuspended in 500 μl permeabilization buffer (PBS, 1% BSA, and 0.2% saponin) on ice for 10 min. Cells were subsequently stained with biotinylated anti- PI(3,4,5)P_3 IgM antibody (Echelon Biosciences) or biotinylated IgM isotype control (BD Biosciences PharMingen) in permeabilization buffer on ice for 30 min. Cells were washed twice with permeabilization buffer, resuspended in the same buffer containing strepavidin-FITC and B220-APC-Cy7, and analyzed by flow cytometry. For receptor occupancy, cells were incubated in 20 μg/ml hen egg lysozyme (Sigma) or PBS on ice for 1 hr. Cells were blocked with anti-mouse CD16-CD32, stained with biotinylated anti-hen egg lysozyme (Rockland), and subsequently stained with strepavidin-PE and anti-B220-APC-Cy7. Mean fluorescence intensities of PBS-treated cells were divided by the corresponding mean fluorescence intensities of HEL-treated cells and multiplied by 100 so that the percentage of receptor occupancy was obtained.

Serum ELISA

Serum samples were collected by retroorbital bleeding. Ninety-six-well high-binding-capacity plates were coated with 10 μg/ml HEL or anti-mouse IgM for 24 hr at 4°C. Plates were blocked for 1–2 hr at RT with blocking buffer (0.5% BSA in PBS). Serum samples were serially diluted in blocking buffer and incubated in coated wells for 2 hr at RT. Plates were washed and incubated with alkaline phosphatase-conjugated anti-mouse IgM or conjugated anti-mouse kappa (Southern Biotech) for 1 hr at RT. Phosphatase substrate (Sigma) was added to wells, and A_{405} was measured with a BioTek Elx808 colorimetric plate reader (BioTek Instruments).

Cell Transfer

Purified B cells were incubated at 1 × 10^7 cells/ml for 10 min at RT in PBS containing 5 μM CFDA-SE (Invitrogen). FCS was added to a final concentration of 10%, and cells were incubated for 15 min at RT. Cells were washed twice with PBS and were injected via tail vein into recipient mice. After 18–26 hr, mice were sacrificed and splenic cells were stained with antibodies against B220 and IgM, and CFSE+ cells were gated and analyzed by flow cytometry.

HEL Injection

Animals were immunized via intraperitoneal (i.p.) injection with either 1 mg HEL in PBS; this was followed 24 hr later with a second 1 mg HEL injection or with a single 5 mg injection. Control mice were injected with PBS alone. Forty-eight hours after the initial injection, animals were sacrificed and spleen suspensions were prepared. For flow cytometric analysis of cellular activation, nonspecific binding was first blocked by preincubating cells with anti-mouse CD16-CD32 blocking antibodies (eBioscience). Cells were then stained with biotin-conjugated anti-mouse CD86 and subsequently stained with strepavidin-PerCP-Cy5.5 (eBioscience), IgM-APC, and B220-APC-AF750 (eBioscience).

Calcium Flux

Two million B cells were resuspended in 250 μl media (DMEM, 10 mM HEPES, and 2.5% FBS). Four μl Fura Red, 2 μl Fluo-4, and 2 μl pluronic acid (Molecular Probes) were added to 1 ml of media. An equal volume of dye mix was added to each cell suspension. Cells were incubated for 45 min at 37°C in the dark, washed with media, and stained with B220-APC. Stained cells were read for 1 min for obtaining a baseline on the flow cytometer and then stimulated with either 10 μg/ml anti-IgM F(ab')$_2$ or 10 μg/ml hen egg lysozyme. Calcium flux was measured by Fluo-4 (530 nm)/Fura Red (685 nm) emission ratiometry for 5 min.

^{3}H-Thymidine Incorporation

Purified B cells were cultured at 1 to 2 × 10^6 cells/ml in round-bottom 96-well plates in 100–200 μl/well of RPMI complete medium in the presence of IL-4 (5 ng/ml) plus LPS (20 μg/ml) Serotype 0111:B4 (Sigma) or anti-IgM F(ab')$_2$ (20 μg/ml). Cells were cultured for 48 hr after which ^{3}H-thymidine was added for the last 16 hr at 1 μCi per well. Cells were harvested with a FilterMate Harvester (PerkinElmer), and the amount of incorporated ^{3}H-thymidine was measured with a MicroBeta Trilux scintillation counter (PerkinElmer).

Bone Marrow Culture

Mixed femoral bone marrow cells were treated with hypotonic solution for red blood cell depletion for 5 min on ice. B cells were purified with anti-B220 MACS beads and cultured at 2 × 10^6 cells/ml in 6-well plates in 15% fetal bovine serum in OptiMEM media containing 10 ng/ml rIL-7, 10 ng/ml SCF, and 10 ng/ml Flt3-L. After 6 days, nonadherent cells were collected in fresh media, labeled with CFSE, and recultured in media containing IL-7 alone, 10 μg/ml anti-IgM F(ab')$_2$, or 1 μg/ml anti-IgM F(ab')$_2$ and incubated for 3 days. Cells were harvested and stained with 7AAD and antibodies to CD86 and B220.

Statistical Method

Student's t test was used for determining statistically significant differences between samples.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/immunity/supplemental/S1074-7613(09)00453-1.

ACKNOWLEDGMENTS

We thank members of the Rickert lab for discussions and critical reading of the manuscript, J. Cambier for helpful discussions, E. Schuman for administrative assistance, J. Browne for assistance with the figures, and the BIMR animal facility for animal husbandry. This work was supported by National Institutes of Health grant AI041649.

Received: October 6, 2008
Revised: June 15, 2009
Accepted: August 21, 2009
Published online: November 5, 2009

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